Supporting Information

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SI Materials and Methods

Antibodies. Antibodies were purchased as follows: Cox4 (Abcam; ab14744), Cox5a (Abcam; ab110262), Cox5b (Abcam; ab110263), Cor1 (Abcam; ab110252), actin (Santa Cruz; sc-1615), HIGD1A (Proteintech; 21749-1-AP), Flag (Sigma; F1804), Vdac (Abcam; ab14734), and Alexa 488- and Alexa 568-labeled secondary antibodies (Invitrogen; A-11001, A-11034, and A-11004). Polyclonal antibodies against Higd1a were generated by immunization with a peptide corresponding to rat Higd1a amino acid sequence (amino acids 2–18, STNTDLSLSSYDEGQGSC) in rabbit.

Cell Culture. Cardiomyocytes obtained from 1-or 2-d-old Wister rats were prepared and cultured in DMEM (Invitrogen) containing 10% (vol/vol) FBS and 100 units/mL penicillin-streptomycin-glutamine. Cells were cultured under 5% CO_2 at 37 °C. For hypoxic exposure, cells were placed in a MCO-5M multigas incubator (Sanyo).

Microarray Data Analysis. Data analysis and normalization were previously mentioned (1). In brief, data analysis was performed using the GeneSpring GX 12.5 bioinformatics software (Agilent Technologies), using the latest gene annotations available. One-way ANOVA test was applied to the filtered gene list, resulting in a group of genes with significant differences. A hierarchical clustering method (heat map constructions) was used to group genes on the basis of similar expression patterns over all samples. Comparisons of the lists of up-regulated genes among them were performed by Venn diagrams.

Constructs. The coding sequence of rat *Higd1a* (NM_080902.3) was amplified by PCR from neonatal rat heart cDNA and cloned into pENTR/D-TOPO (Invitrogen). Next, carboxyl terminus Flag tag was fused by PCR. For adenoviral construction, we used the ViraPower Adenoviral Expression system (Invitrogen) and the BLOCK-iT Adenoviral RNAi Expression system for shRNA (Invitrogen). For shRNA construction, shRNA oligonucleotides were designed as synthetic duplexes with overhanging ends identical to those created by restriction enzyme digestion site. Each oligonucleotide containing a target sequence was subcloned into pENTR-U6 and recombined into pAd/BLOCK-iT-DEST. The target of shRNA for *Higd1a* is the 5′-UTR. The sequences used are as follows: shRNA for *Higd1a*, ccgaagactcttcaagaaa and shRNA for *LacZ*, ctacacaaatcagcgattt. The adenoviral particles were produced by transfection in 293A cells.

Immunoprecipitation. Cardiomyocytes were collected in isotonic buffer (pH 7.4, 25 mM Hepes, 250 mM sucrose, 1 mM EDTA) and mitochondrial pellets were isolated from cell cultures as described (2) with slight modification. Mitochondrial pellets were lysed with lysis buffer (pH 7.4, 30 mM Mops, 150 mM NaCl, 1 mM EDTA, 5% (vol/vol) glycerol containing 1% *n*-dodecyl-β-D-maltoside (DDM)) for 30 min at 4 °C. After a clarifying spin, samples were incubated with individual antibodies for 2 h at 4 °C followed by addition of protein G Sepharose (GE Healthcare) for 30 min at 4 °C. After washing, the bound proteins were eluted with SDS/PAGE sample buffer.

Immunoblotting. The 200×10^4 cardiomyocytes treated under 1% oxygen were lysed in pH 7.4, 30 mM Mops, 150 mM NaCl, 1 mM EDTA, 5% (vol/vol) glycerol, 1% DDM, protease inhibitor mixtures (Amresco). After centrifugation for 5 min at $21{,}900 \times g$ to remove insoluble material, lysates were assessed for protein levels using the BCA method (Pierce) and 4 μg of soluble protein from each was resolved on 12% Bis-Tris gels 160 V for 45 min. Gels were transferred to PVDF and Western blotted.

Immunofluorescence Assay. Cardiomyocytes were seeded on collagen-coated 35-mm glass dishes (Asahi Techno Glass). After 24 h from seeding, the cells were washed once with PBS and fixed with 4% paraformaldehyde for 10 min. The cells were permeabilized with 100% methanol for 10 min and then immunostained with anti-Higd1a polyclonal antibody and anti-Cox4 monoclonal antibody. For secondary reactions, Alexa 488- or 568-labeled secondary antibodies (Invitrogen) were used.

The cells infected with adenovirus encoding carboxyl terminus Flag-tagged Higd1a (adHig), were treated with 50 nM of Mito-Tracker Red for 30 min, washed once with prewarmed PBS, and fixed using the same method described as above. For lebeling, anti-Flag M2 monoclonal antibody (Sigma-Aldrich) was used. Fluorescence images were recorded with an Olympus FV1000D confocal microscope using a PL APO 60×, 1.35 N.A. oil immersion objective lens (Olympus).

Pull-Down Assay. Cytochrome *c* oxidase from bovine heart was purified by the method reported previously (3–5). Recombinant MBP-Higd1a (5 μg) and highly purified cytochrome *c* oxidase (hpCcO) (20 μg) were incubated at 25 °C for 30 min in the presence of 0.2% *n*-decyl-β-D-maltoside (DM), and amylose resin was added at 4 °C for 1 h. After washing, bound proteins were eluted by SDS/PAGE sample buffer.

Blue Native PAGE. A total of 10 μg of mitochondrial pellets, as measured by using the Bradford method, from cardiomyocytes was solubilized, containing 2% (wt/vol) digitonin. Solubilized samples were incubated on ice for 10 min and centrifuged for 30 min at $100{,}000 \times g$ at 4 °C. Supernatants were added to Coomassie G-250 and resolved on a 4–16% Native PAGE Bis-Tris gel (Invitrogen). Initially run for 30 min with a constant voltage of 150 V using a cathode buffer (pH 7.0, 50 mM tricine, 7.5 mM imidazole, and 0.02% Coomassie G-250) and anode buffer (pH 7.0, 7.5 mM imidazole). Next, the cathode buffer was exchanged into the light cathode buffer (pH 7.0, 50 mM tricine, 7.5 mM imidazole, 0.002% Coomassie G-250) and run for another 75 min with a constant voltage of 150 V. After electrophoresis, proteins were transferred to PVDF membrane (0.45 μm, Millipore) using Mini Trans-Blot Cell Assembly (BIO-RAD, pH 8.3, 25 mM tricine, 192 mM glycine) with a constant voltage of 30 V.

Recombinant MBP-Higd1a and hpCcO were incubated at 25 °C for 30 min in the presence of 0.2% DM. The mixed solution was resolved by the same electrophoresis method and transferred to PVDF membrane by semidry method (pH 8.3, 25 mM tricine, 192 mM glycine, 20% (vol/vol) methanol, 0.04% SDS) for 60 min with a constant voltage of 15 V. Then, membranes were washed with 8% (vol/vol) acetic acid followed by deionized-distilled water. Each antibody was conjugated and detected with a CCD camera-based detection system (ImageQuant LAS-4000, GE Healthcare).

Measurement of Intact Cellular Respiration. Oxygen consumption rates were measured by XF96 Extracellular Flux Analyzer (Seahorse Bioscience) in unbuffered DMEM containing 25 mM glucose, 2 mM-L-glutamine, and 1 mM sodium pyruvate under basal conditions and in response to 1 μM oligomycin A, 0.5 μM fluorocarbonyl cyanide phenylhydrazone (FCCP) and 100 nM rotenone + 100 nM antimycin A (Sigma). For measurement, 6×10^4 cardiomyocytes were treated with each adenovirus (adLacZ or adHigd1a or shLacZ or shHigd1a).

Measurement of ATP Synthesis in Permeabilized Cardiomyocytes. Speed of ATP synthesis in cardiomyocytes (1.2×10^4 cells per well for shRNA-treated groups and 7.5×10^3 cells per well for adLacZ or adHigd1a-treated groups) permeabilized with 2% (wt/vol) digitonin was measured as described previously (6). In brief, after addition of assay buffer, the luminescence (produced ATP) was measured every minute for 15 min using ATP bioluminescence assay kit CLS II (Roche). Every time point of data was divided by a value of ATP production at 10 min in shLacZ or adLacZ groups. ATP production at 10 min in shLZ or adLZ was regarded as 1.0.

Cell Viability. A total of 5×10^5 cells of cardiomyocytes were transfected with either shHigd1a (shHig) or shLacZ (shLZ), cultured for 60 h, and then subjected to hypoxic conditions (1% oxygen) for another 24 h. After hypoxia, the cells were stained with 2 μg/mL propidium iodide (Sigma) and 2 μg/mL Hoechst 33342 (Dojin Chemical) at 37 °C for 30 min. The cells were analyzed using IN Cell Analyzer 6000 (GE Healthcare).

Structure Modeling. A docking model of the CcO and Higd1a was constructed as follows: The initial docking model was created in COOT (7) using the coordinates for the COX (Protein Data Bank, PDB: 3ABM, removal of TG1 and TG3) and the HIGD1A (PDB: 2LOM, model 6 of 20 structures) and then refined by energy minimization without any structure factor terms by the Crystallography and NMR system (8, 9).

Statistical Analyses. The comparison between two groups was made by *t* test (two tailed). For MASC assay, comparison was made by repeated two-way ANOVA. A value of $P < 0.05$ was considered statistically signifcant. Data represent mean ± SEM.

1. Kioka H, et al. (2014) Evaluation of intramitochondrial ATP levels identifies G0/G1 switch gene 2 as a positive regulator of oxidative phosphorylation. *Proc Natl Acad Sci USA* 111(1):273–278.
2. Klement P, Nijtmans LG, Van den Bogert C, Houstěk J (1995) Analysis of oxidative phosphorylation complexes in cultured human fibroblasts and amniocytes by blue-native-electrophoresis using mitoplasts isolated with the help of digitonin. *Anal Biochem* 231(1):218–224.
3. Tsukihara T, et al. (1995) Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 A. *Science* 269(5227):1069–1074.
4. Shinzawa-Itoh K, et al. (1995) Effects of ethyleneglycol chain length of dodecyl polyethyleneglycol monoether on the crystallization of bovine heart cytochrome c oxidase. *J Mol Biol* 246(5):572–575.
5. Yoshikawa S, Choc MG, O'Toole MC, Caughey WS (1977) An infrared study of CO binding to heart cytochrome c oxidase and hemoglobin A. Implications re O2 reactions. *J Biol Chem* 252(15):5498–5508.
6. Fujikawa M, Yoshida M (2010) A sensitive, simple assay of mitochondrial ATP synthesis of cultured mammalian cells suitable for high-throughput analysis. *Biochem Biophys Res Commun* 401(4):538–543.
7. Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66(Pt 4):486–501.
8. Brunger AT (2007) Version 1.2 of the Crystallography and NMR system. *Nat Protoc* 2(11):2728–2733.
9. Brünger AT, et al. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* 54(Pt 5): 905–921.
10. Acín-Pérez R, Fernández-Silva P, Peleato ML, Pérez-Martos A, Enriquez JA (2008) Respiratory active mitochondrial supercomplexes. *Mol Cell* 32(4):529–539.

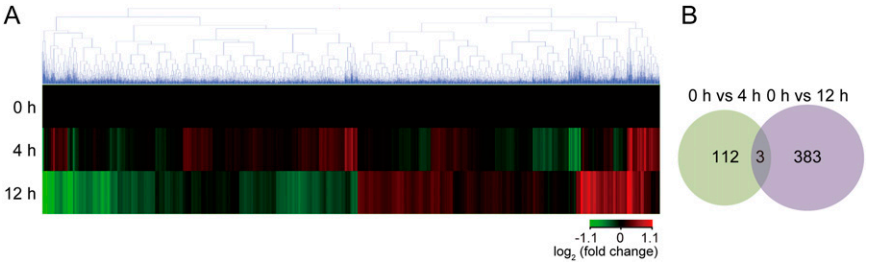

Fig. S1. Three genes were identified by microarray analysis using hypoxia-treated cardiomyocytes. (*A*) Hierarchical clustering image representing 2,598 genes exhibited significantly ($P < 0.05$; ANOVA) different expression levels at each of three time points (0, 4, and 12 h). (*B*) The Venn diagrams represented the overlap of genes that were up-regulated (>2.0-fold change up-regulated) at 4 h and genes that down-regulated (<1.2-fold change) by 12 h compared with 0 h, respectively.

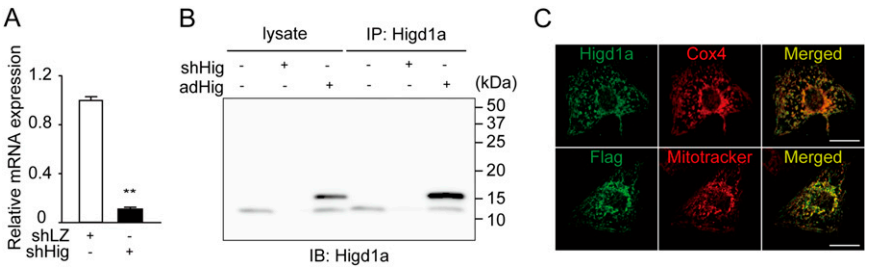

Fig. S2. Validation of our raised antibody against Higd1a. (*A*) Rat cardiomyocytes transfected with either shLacZ (shLZ) or shHigd1a (shHig) were analyzed by quantitative RT-PCR. *Higd1a* mRNA level was normalized by *Actb*. Data represent means ± SEMs ($n = 8$); $^{**}P < 0.01$, compared with control shLZ. (*B*) Validation of our established antibody for rat Higd1a. Immunoprecipitation was followed by immunoblot analysis of cardiomyocytes treated with shHig or adenovirus encoding carboxyl terminus Flag-tagged Higd1a (adHig). (*C*, *Upper*) Endogenous Higd1a was stained in cardiomyocytes with anti-Higd1a (green) and anti-Cox4 (red). (*Lower*) Cardiomyocytes transfected with adHig were stained with anti-Flag (green) and MitoTracker (red). The established antibody specifically recognizes neonatal rat Higd1a, which cannot be detected by commercially available Higd1a antibody (Scale bars, 20 μm.)

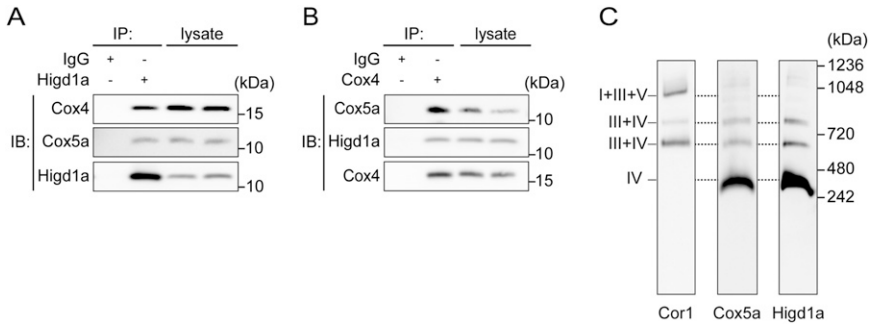

Fig. S3. Higd1a associated with CcO in rat cardiomyocytes. (*A*) Mitochondrial fractions isolated from rat cardiomyocytes were lysed and immunocaptured using an anti-Higd1a antibody and then immunoblotted with anti-Cox4 or anti-Cox5a antibodies to detect CcO subunits. (*B*) Reciprocal coimmunoprecipitation with an anti-Cox4, one of the subunits of CcO. (C) Mitochondrial fraction from rat cardiomyocytes was solubilized in 2% (wt/vol) digitonin and resolved by BN-PAGE, followed by immunoblotting with anti-Cox5a to detect complex IV or anti-Cor1 to detect complex III or anti-Higd1a. Higd1a integrated into the CcO, as it behaved similar to Cox5a, not to Cor1.

Fig. S4. Alignment of human Higd1a and bovine Higd1a with ClustalW (v 2.1). Black boxes, identical residues in both species; gray boxes, similar amino acids. Sequences of Higd1a are almost matched between human and bovine.

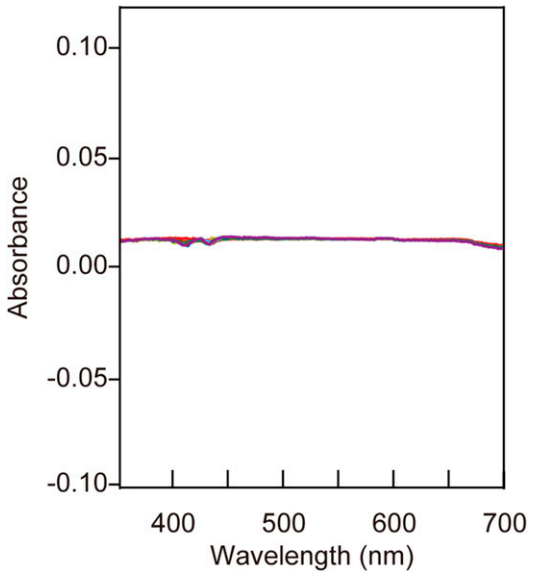

Fig. S5. Supporting data for Fig. 2. Optical absorption intensity changes of oxidized hpCcO spectra are plotted at 1 min (red), 5 min (brown), 10 min (dark yellow), 15 min (green), 20 min (light blue), 25 min (blue), and 30 min (purple) after adding MBP.

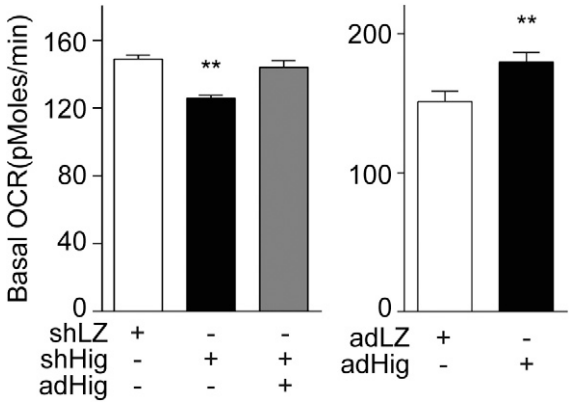

Fig. S6. Supporting data for Fig. 3. Basal oxygen consumption of rat cardiomyocytes transfected with either shLZ for control, shHig, or both shHig and adHig (n = 20 for each group).

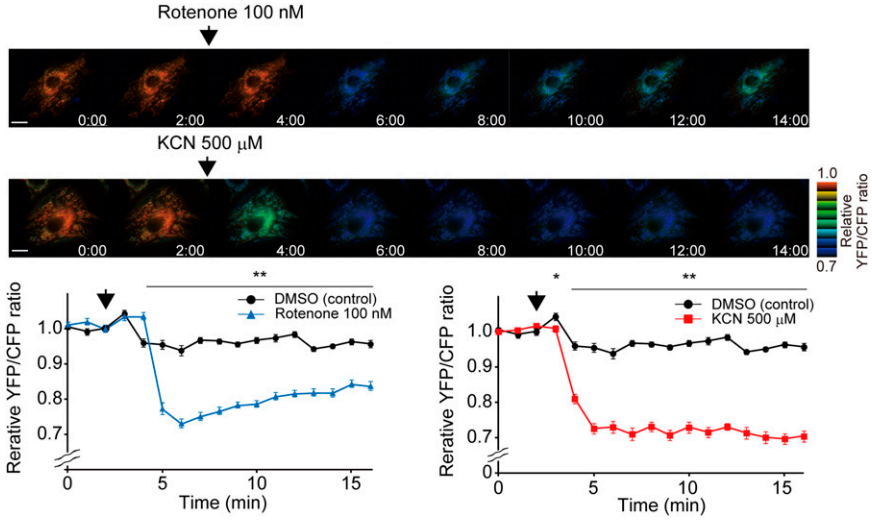

Fig. S7. Mit-ATeam assay reflects the function of complex I or IV. Mit-ATeam assay in rat cardiomyocytes is highly sensitive to assess the effect of inhibitors of rotenone (inhibitor of complex I) or KCN (inhibitor of complex IV) on mitochondrial oxidative phosphorylation. *Lower* line graphs represent YFP/CFP emission ratio plots of Mit-ATeam in rat cardiomyocytes. Rotenone (100 nM) or KCN (500 μM) was added at 2 min (arrowhead; n = 8), respectively. All measurements were normalized to the YFP/CFP emission ratio at 0 min. Data represent means ± SEMs. (Scale bars, 20 μm.)

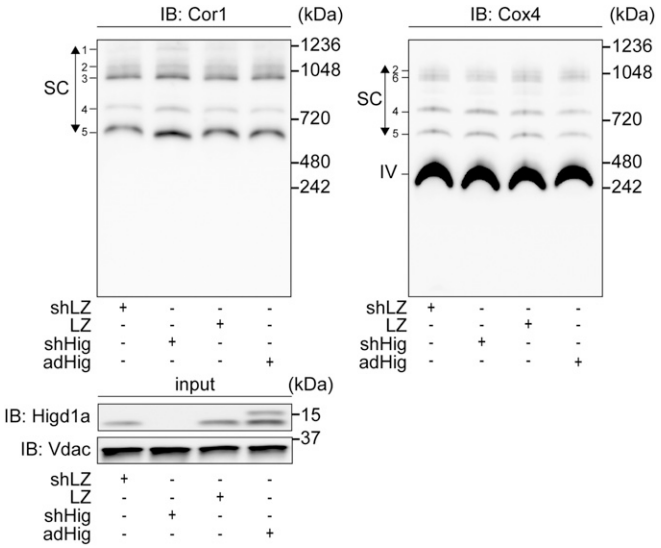

Fig. S8. Higd1a does not affect respiratory supercomplex formation. Mitochondrial fraction from rat cardiomyocytes transfected with corresponding viruses was solubilized in 2% (wt/vol) digitonin and 8 μg of each fraction was subjected to BN-PAGE, followed by immunoblotting with anti-Cox4 to detect complex IV or anti-Cor1 to detect complex III. The numbers, which are located on the *Left* side of the two *Upper* Western blots, represent respiratory supercomplex, specifically: 1 (I + III + V); 2 (I + II + III + IV); 3 (I + III + V); 4 (III + IV); 5 (III + IV); and 6 (I + III + IV) (10). *Lower* blot represents SDS/PAGE of inputs. Loading control, blotted with anti-VDAC antibody.

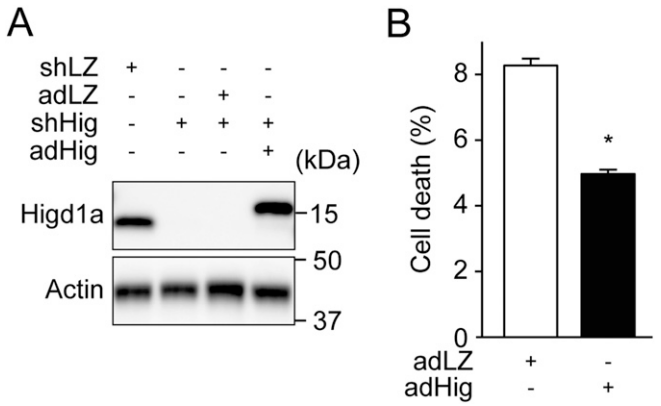

Fig. S9. Supporting data for Fig. 4*B*. (*A*) Immunoblotting of Higd1a treated with corresponding adenoviruses. (*B*) The cell viability was increased by exogenous adHig compared with adLZ under hypoxia for 14 h. Data represent means ± SEMs. $*P < 0.05$, $**P < 0.01$, compared with control (adLZ or shLZ).

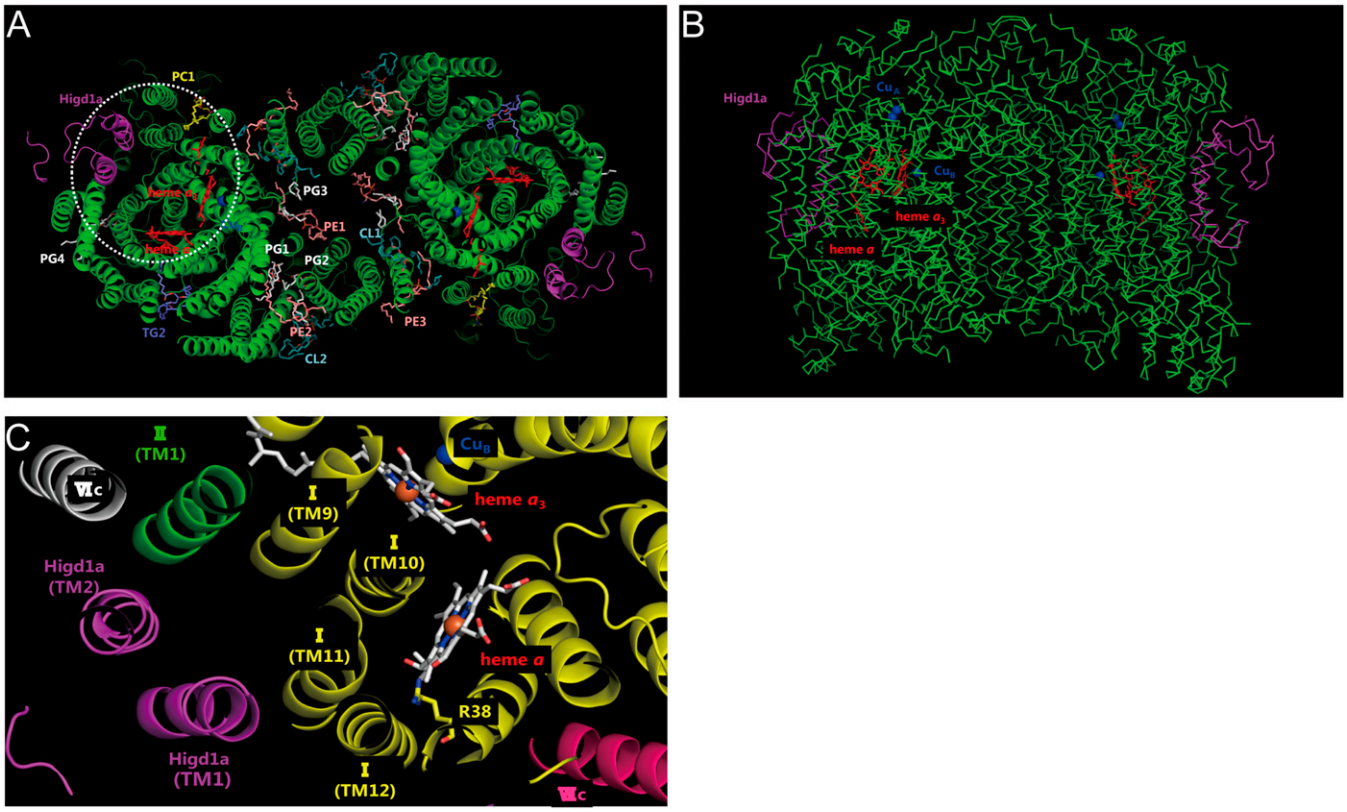

Fig. S10. Docking simulation between CcO (PDB accession code: 3ABM, removal of TG1 and TG3) (the Cα backbone trace: green) and Higd1a (PDB accession code: 2LOM, model 6 of 20 structures) (magenta). Higd1a integrated into the cleft of CcO, just outside of the active center. (*A*) Top view. (*B*) Side view. CL 1–2, cardiolipin (light blue); PC, phosphatidylcholine (yellow); PE 1–3, phosphatidylethanolamine (pink); PG 1–4, phosphatidylglycerol (white); and TG 2, triglyceride. (*C*) Enlarged image of docking simulation (dotted white circle in *A*). The TM1 of Higd1a (magenta) is near the TM11 and TM12 of subunit I (yellow) and it is likely that the binding of Higd1a affects the structure of R38 (yellow) and formyl group of heme *a*.